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RESEARCH ARTICLE

A time-lapse approach to examine chromium and nickel effects on wound healing *in vitro*

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Abstract

Chromium and nickel cause allergic contact dermatitis, a common biological skin response to sensitizing agents. This study used a conventional *in vitro* wounding model to study the impact of sensitizing agents on the innate immune response of human keratinocytes. Experiments were designed to evaluate the involvement of specific Toll-like receptors and metalloproteinases as effectors molecules downstream, at a molecular level. Further, keratinocytes were co-cultured with monocytes (THP-1 cells) to reproduce an inductive stimulus on monocytes made by metals. Human keratinocytes (HaCat) were grown on plates covered with collagen type I, chemically treated, and then mechanically injured with a sterile pipette tip. Restoration of the monolayer integrity was monitored by time-lapse video microscopy. Effector gene expression was evaluated by real-time PCR. The presence of chromium significantly dropped the rate of wound closure, while nickel-induced hyper-proliferation ended in an acceleration of the healing process, an event that does not occur *in vivo*. This latter outcome led to considering nickel as an unsuitable example for use in the experimental model. Focusing solely on the chromium aspect of this study, RNA profiles of selected molecular markers were generated to ascertain if the detrimental stimulus from chromium was eliminated or persisted both in keratinocytes alone and/or during co-cultures of keratinocytes and monocytes. Monocytes accelerated the process of wound repair. This *in vitro* experimental model highlighted the involvement of innate immunity in response to chromium and might be useful for test molecules of therapeutic interest for the treatment of skin lesions. However, the experience with nickel reveals that there are limitations to the utility of this wound model system after all.

Keywords: Wound healing, TLRs, MMPs, chromium

Introduction

Nickel (Ni) and chromium (Cr) are important skin sensitizers, causing allergic contact dermatitis (ACD), an inflammatory process of the skin (Forte et al., 2008). In industrialized countries, Ni is the most frequent cause for contact hypersensitivity reactions, with an estimated rate of 39% of young women being sensitized. In contrast to Ni, Cr is most commonly a cause of occupational ACD. Hexavalent salts, which are more soluble than trivalent salts, penetrate more easily through the skin where they are reduced to Cr³⁺, considered to be the sensitizing agent.

ACD is a form of delayed-type hypersensitivity reaction that results from an immune response to small chemical substances, the haptens, penetrating into the skin. ACD is mediated by T-lymphocytes that specifically recognize hapten-modified peptides (Saint Mezdard et al., 2004). T-Lymphocytes are known to recognize antigens as short processed peptides bound to major histocompatibility complex (MHC) molecules on the surface of antigen-presenting cells (APC) (Netea et al., 2005; Hussein et al., 2009). The epidermis provides the first line of defense from external insults through active immunologic events. Human keratinocytes express functional Toll-like receptors (TLRs) involved in innate and acquired

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immune responses (Köllish et al., 2005; Miller and Modlin, 2007). Keratinocytes are activated by haptens and initiate inflammatory processes by releasing specific signals consisting of cytokines and growth factors. This cytokine network seems necessary for efficient hapten handling by APC, mostly Langerhans cells (LC), and for T-lymphocyte priming in the regional lymph nodes (Saint Mezdard et al., 2004; Sugita et al., 2006). Epidermal keratinocytes are the major target of these cytokines and their interaction with monocytes play an important role in the inflammatory skin response to metal exposure.

The TLR are a family of cellular receptors that are critical in the innate immune response because of their ability to recognize pathogen-associated molecular patterns (PAMPs) such as bacteria, viruses, and fungi (Rahman et al., 2009). While the role of TLR(s) in pathogen recognition has been expeditiously advanced in recent years, investigations into the function of TLR(s) in non-infectious tissue injury have just begun (Rahman et al., 2009). In fact, endogenous ligands have been described and may be considered damage-associated molecular patterns (DAMPs) (Bianchi, 2007). TLR(s) may be involved in the patho-physiology underlying the development of aberrant IgE production and the associated dermatitis. These TLR(s) are usually associated with antigen-presenting cells and have the capacity to affect adaptive immunity (T- and B-lymphocyte-mediated immune responses) by the cytokine patterns that TLR(s) trigger, which subsequently shape T-helper (T_H)-1- vs T_H -2 lymphocyte development during antigen presentation (Gaspari, 2005).

Tissue damage provides mitogenic signals to adjacent cells that can, in turn, induce cell migration (for tissue repair) and remodeling processes that are mediated by induction of metallo-proteinases and angiogenic factors. Matrix metalloproteinases (MMP) are a family of protein-cleaving enzymes that degrade extracellular matrix (ECM) and basement membrane components. Alteration of the ECM is integral to the resolution of wound healing but also has implications in inflammation (Gill and Parks, 2008). A major difference between acute and chronic wounds is the imbalance of proteinase inhibitors and activity, regulating degradation and regeneration of intercellular matrix proteins. ECM proteolysis executed by MMP may be a pre-requisite for proper wound repair and accurate tissue regeneration. MMP and their inhibitors have many responsibilities in the regulation of inflammation, and this includes the control of chemokine activity, the establishment of chemotactic gradients, and the regulation of inflammatory-epithelial cell adhesion receptor interactions (e.g. intercellular adhesion molecule-1, ICAM-1) (Hartenstein et al., 2006; Gill and Parks, 2008).

In this research, we analyzed the primary keratinocytes responses to sensitizing agents (nickel and chromium), at cellular and molecular levels. To this end, we used an established *in vitro* model of wound healing looking at the capability to restore the integrity of the monolayer and the role of innate elements that recognize and mediate sterile inflammation. A keratinocyte cell line (HaCat) was grown

on plates coated with collagen and mechanical damage was performed (using sterile pipette tip). The morphology was constantly monitored by time-lapse microscopy, allowing an accurate analysis of images to calculate reductions in lesion margins over time. Molecular profiles of TLR(s), MMP(s), and ICAM-1, key effectors in the healing process, were also examined. Additionally, co-cultures of HaCat with THP-1 monocytes were performed to verify the effects of the interactions between keratinocytes and monocytes, in the presence of Cr.

Materials and methods

Cells culture conditions and chemicals

HaCat cells (obtained from 'Istituto Zooprofilattico di Brescia', Brescia, Italy), a spontaneously transformed non-tumorigenic human keratinocyte cell line, were cultured in Dulbecco's Minimal Essential Medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS) and 1% penicillin-streptomycin (complete medium) at 37°C in a 5% CO₂ incubator. For experiments, cells were harvested via trypsin-EDTA treatment, seeded into 35 mm² culture plates that had been pre-coated with collagen Type I (Sigma, St. Louis, MO) at a density of 1×10^6 cells and grown until $\approx 80\%$ confluence. The culture medium was then removed and replaced by either fresh medium alone (control) or medium containing nickel (Ni [as NiSO₄]) or chromium (Cr [as K₂Cr₂O₇]), both from Sigma, Milan, Italy. THP-1 cells, a human monocytic cell line (obtained from ATCC-LGC, Venice, Italy), were grown in RPMI medium supplemented with 2×10^{-3} M glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 10% heat-inactivated FCS in 25 cm² flasks maintained at 37°C in a 5% CO₂ incubator. All culture medium and supplements were from Lonza (Milan, Italy).

For the experiments, cells were added to the injured keratinocytes monolayer (at a ratio of 1:3 monocytes:keratinocytes) and co-incubated for periods of up to 72 h. The Ni and Cr were used at a concentration of 10^{-5} M and 10^{-6} M, respectively. These concentrations were chosen as they were previously shown to be sub-cytotoxic in previous studies (see Cammarota et al., 2006; Perfetto et al., 2007).

Wound healing assay: Time-lapse video microscopy

For the wound assay, 1×10^6 keratinocytes were grown to confluence on collagen-coated 35 mm² culture plates. Cells were then serum starved for 12 h before sensitizing agents were applied. Pre-treatment was performed for 72 h with 10^{-5} M Ni or 10^{-6} M Cr in complete medium. At the end of the period, scratches were created using a sterile pipette tip. After washing away any non-adherent cells, cultures were re-fed medium supplemented with 2% FCS, in the presence (indicated as Cr + Cr) or not of metal as before, to limit cell proliferation. Control wells (indicated as CTR) were treated similarly, but *without* pre-treatment with either metal in the medium or addition after wounding (wounded cells/no metal). For the co-culture

experiments, keratinocytes were chemically pre-treated for 72 h, then mechanically injured and incubated with THP-1 cells (at a ratio of 1:3 HaCat:THP-1) with (Cr+Cr) or without Cr. Again, control cells were kept under the same conditions, but in the absolute absence of Cr (no pre-treatment nor addition after wounding).

To observe wound closure, plates were monitored for 72 h using a time-lapse video microscopy apparatus (Okolab, Naples, Italy [www.oko-lab.com]). Time-lapse video microscopy used an optic microscopic station, equipped with a 'stage incubator' where temperature and the atmosphere were kept at 37°C and 5% CO₂, respectively (Fotos et al., 2006). Phase-contrast pictures of the wound margins were taken every 15 min throughout the experiments (72 h). Fields were selected with micrometric precision (in a three-dimensional manner, axes xyz). The recorded images were analyzed using Image pro-plus 5.1 software (Media Cybernetics Inc., Bethesda, MD) that measured the reduction in cleared space between the wound edges in the treated vs control samples.

Injury induction for molecular experiments

To reproduce skin damage *in vitro* and to be sure that the extraction of RNA was made from injured cells, the mechanical damage induced to the cultures was very extensive. With a sterile plastic yellow tip, several parallel 'grooves' were inflicted upon the monolayers. To qualify as extensive, the damage must have involved at least 60% of the cells (Lan et al., 2010).

MTT proliferation assays

Serum-starved HaCat cells (2×10^3 in a final volume of 200 µl serum-free DMEM/well) treated or not with Ni and/or Cr were grown in 96-wells flat bottom microplates at 37°C, under 5% CO₂, for 48 h. Assays were then carried out using an MTT proliferation Kit (Roche Molecular Biochemical, Milan, Italy). Briefly, at the end of the incubation, 20 µl of MTT labeling reagent was added (final concentration 0.5 mg/ml) to each well. After 4 h, 100 µl of kit-provided solubilization solution was added to each well, and the plate incubated (with gentle agitation) for 1 h at room temperature to permit cleavage of MTT to purple formazan crystals (derived from yellow tetrazolium salt) generated in the metabolically active cells. The formazan crystals formed were solubilized (by addition of 1M HCL in isopropanol for 3 min) and the resulting colored solution quantified using a microplate reader (ELISA, Bio-Rad Laboratories, Milan, Italy) at 570/655 nm wavelengths. Cell proliferation or any reduction of cell viability was expressed as a percentage of proliferation comparing treated cells vs control cells (serum-free cells). Positive control cells were cultured with 10% FCS.

Real-time PCR

In order to identify potential mechanisms for the observed wound closure hold-up, total RNA was extracted from Cr-treated HaCat cells at different time intervals. Similar gene expression studies with co-cultures were not done.

This is because performing gene expression studies with co-cultures presents a difficult challenge, i.e., there needs to either physically be a good separation of the two populations or some mechanism to distinguish the source of the gene(s) being analyzed.

After the wounding, damaged cells were removed by extensive washes to prevent their contaminating signals coming from viable, activated cells by that from these damaged/dead cells. Total RNA from the viable cells was then isolated by a High Pure RNA Isolation Kit (Roche Diagnostics, Milan, Italy) from HaCat cells and transcribed by reverse transcriptase (Expand Reverse Transcriptase, Roche) at 42°C for 45 min according to the manufacturer's protocol. The resulting cDNA was subjected to real-time polymerase chain reaction (PCR) analysis by rapid cycling in glass capillaries with a thermocycler (Light-Cycler; Roche). The reaction was performed in a final volume of 20 µl with Light-Cycler-DNA Master SYBR Green I (Roche) containing nucleotides, Taq DNA polymerase, buffer, SYBR Green I dye and 0.01 M MgCl₂. cDNA (2 µl), primers and sterile H₂O were added. The sequences of the PCR primers were shown in Table 1.

The amplification protocol consisted of an initial denaturation step at 95°C for 3 min. The cycles used for the primers were: 95°C for 10 s, 55°C for 30 s, for 40 cycles for β -actin, MMP-2, TLR-2, and TLR-4; 95°C for 10 s, 60°C for 30 s, for 40 cycles for 18S, MMP-13, TLR-7, and TLR-9; and, 94°C for 1 min, 61°C for 2 min, 72°C for 3 min, for 34 cycles for ICAM-1. The final melting curve was performed from 55–95°C. SYBR Green I fluorescence was monitored at the end of each cycle to assess the amount of PCR product formed. All reactions were carried out in triplicate, and the relative expression of specific mRNA was determined by normalizing to β -actin (MMP-2, TLR-2, and TLR-4) and 18S (MMP-13, TLR-7, TLR-9, and ICAM-1). The specificity of the amplification products was verified by electrophoresis over a 1.4% agarose gel stained with 1 µg ethidium bromide/ml. All data are

Table 1. Primers used in real-time PCR.

	Primer
TLR-2	Forward 5' gCC TCT CCA Agg AAg AAT CC 3'Reverse 5' TCC TgT TgT Tgg ACA ggT CA 3'
TLR-4	Forward 5' AAg CCg AAA ggT gAT TgT Tg 3' Reverse 5' CTg AgC Agg gTC TTC TCC AC 3'
TLR-7	Forward 5' CTg ACC ACT gTC CCT GAG 3' Reverse 5' AAC CCA CCA gAC AAA CCA 3'
TLR-9	Forward 5' ATT CTg ACT TTg CCC ACC Tg 3' Reverse 5' gCT gAg ggA CAg ggA TAT gA 3'
MMP-2	Forward 5' gCC gCC TTT AAC Tgg AgC AA 3' Reverse 5' TTC CAg gCA TCT gCg ATg Ag 3'
MMP-13	Forward 5' TCC CAg gAA TTg gTg ATA AAg TAg A 3' Reverse 5' CTg gCA TgA CgC gAA CAA TA 3'
ICAM-1	Forward 5' CgT gCC gCA CTg ACC Tgg AC 3' Reverse 5' CCT CAC ACT ACT TCA CTg TCA CCT 3'
18S	Forward 5' CTg CCC TAT CAA CTT TCg ATg gTA g 3'Reverse 5' TTT CTC Agg CTC CCT CTC 3'
β -Actin	Forward 5' gCg AgA AgA TgA CCC AgA TC 3'Reverse 5' ggA TAg CAC AgC CTg gAT Ag 3'

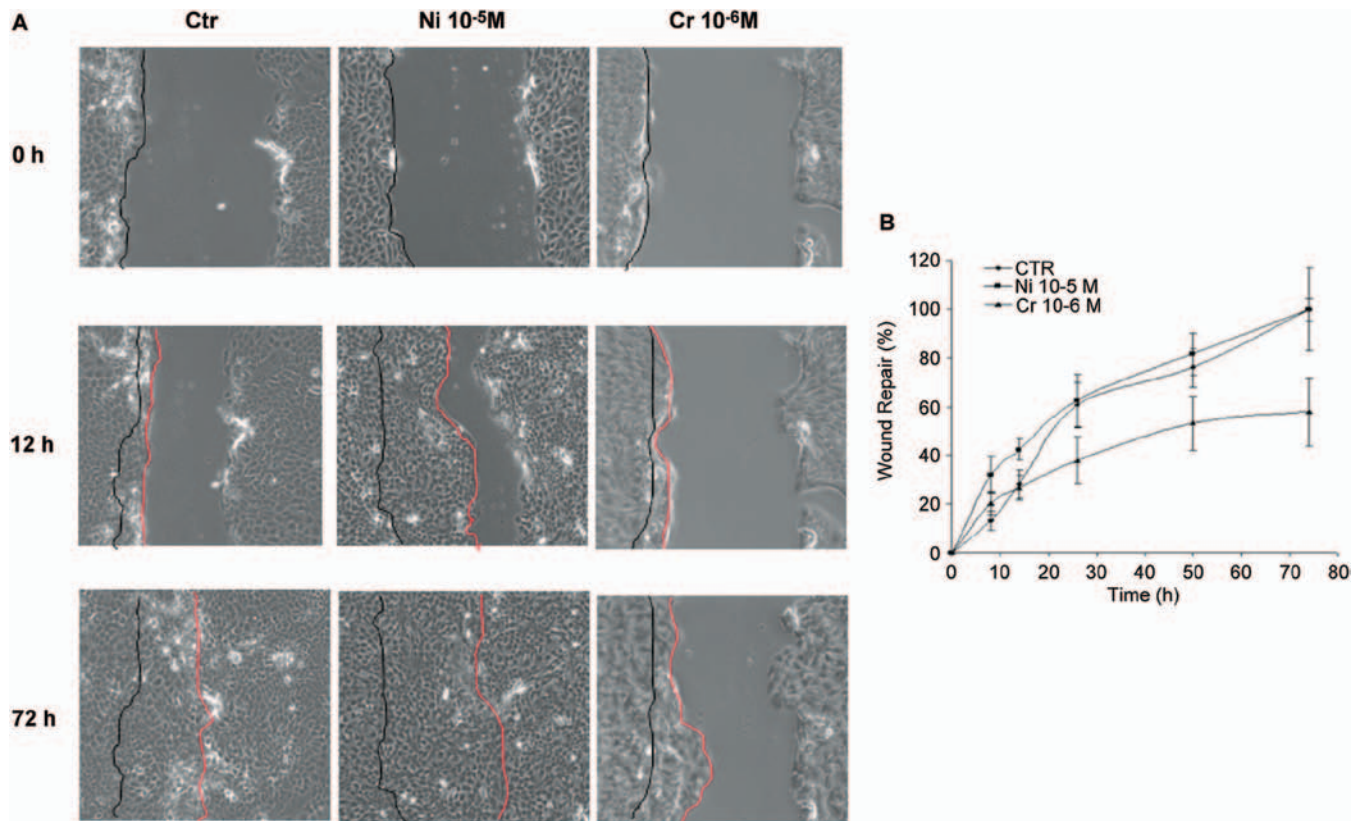


Figure 1. Effects of sensitizing agents on scratch wound healing. Effects of chromium (10^{-6} M) and nickel (10^{-5} M) on scratch wound healing of HaCat cell confluent monolayer. (a) Phase-contrast time-lapse of scratched HaCat monolayer at 0, 12, and 72 h after scratching. In the presence of Cr, the rate of wound closure was significantly decreased and closure was far from complete up to 72 h post-wound initiation. On the contrary, Ni-treated cells seemed to heal as well as the controls (i.e., cells that were wounded but received neither metal pre-/post-wounding). (b) Wound repair (%) of metal-treated cultures (with respect to control). Data represent the mean ($\pm$ SD) of at least four fields of view in three independent experiments. Cr-treated group was significantly different from control ($p < 0.05$).

presented as the ratio between the target gene expression and the gene expression in unstimulated conditions (Schmittgen et al., 2000). The fold-change in test gene mRNA was calculated based upon the efficiency of each primer and extrapolating from a dilution curve (between 80–110% and wherein $\Delta\Delta Ct$ was equal to the difference in ΔCt between Cr-treated cells and control), using Bio-Rad iQ5 software (Bio-Rad Laboratories S.r.l, Milan, Italy).

Statistical analysis

All values reported are expressed as means ($\pm$ SE). One-way analyses of variance (ANOVA) were carried out to determine statistical significances (Microsoft Excel 2000, Microsoft Corporation, Redmond, WA). Differences were considered statistically significant at a level of $p < 0.05$.

Results

Wound closure

To evaluate the impact of sensitizers on wound healing, HaCat cells were grown to confluence, treated for 72 h with nickel (Ni; 10^{-5} M) or chromium (Cr; 10^{-6} M), then scratched with a sterile pipette tip. Post-wound healing was monitored thereafter by time-lapse microscopy. The distances between the edges of the injured monolayer

were measured by Image Pro-Plus 5.1 software and the reduction was calculated using the formula $[(D_n - D_0)/D_0] \times 100$, wherein D_n is the distance measured at time n , and D_0 the distance at starting time. Three independent experiments were carried out. In each experiment, at least four fields of view/sample were analyzed.

Scratch wounding is able *per se* to stimulate re-epithelization in control. However, in the presence of Cr, the rate of wound closure was significantly decreased and was far from complete up to 72 h post-wound (Figures 1a and 1b) additional videos available on request). In contrast, Ni induced hyper-proliferation, ending in an acceleration of the healing process, leading to a wound closure very similar to control (i.e., cells that were wounded but received no metal prior to or after wounding), an event that does not occur in ACD lesions *in vivo*.

So, further experiments were carried out using only chromium (Cr), whose *in vitro* results resembled *in vivo* outcome of ACD lesions (Forte et al., 2008). To evaluate the effects of the persistence of the chemical insult, HaCat were treated with Cr (10^{-6} M), then scratched, and the medium replaced with Cr again (Cr + Cr). The presence of Cr after injury slightly further delayed the wound healing process in respect to the pre-treatment alone (Cr) (Figure 2).

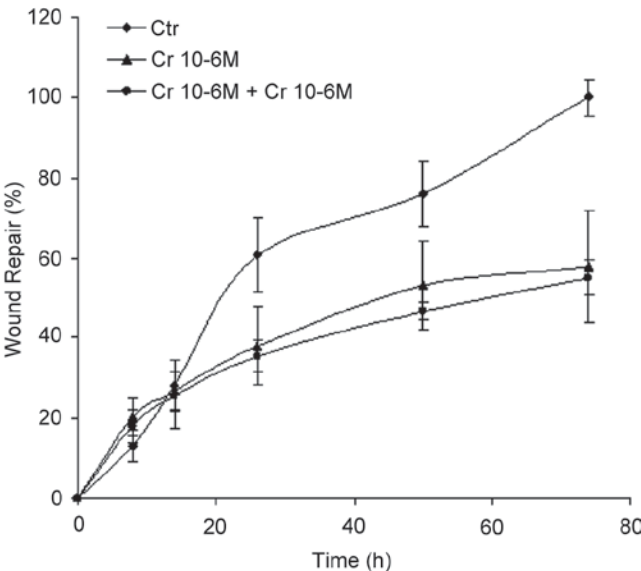


Figure 2. Wound closure in the presence of Cr. HaCat were treated with 10^{-6} M Cr, scratched, and the medium then replaced with Cr again (10^{-6} M). The presence of Cr after injury slightly further delayed the wound healing process with respect to pre-treatment alone.

To evaluate the competence of immune cells in the healing process, HaCat cells were treated with Cr, then scratched before applying the THP-1 cells. The monocytes, visible as bright cells in suspension, were mostly recruited on the scratch borders and accelerated the wound closure in control (Figures 3a and 3b) additional videos available on request). Interestingly, the presence of THP-1 cells helped to close the wound where Cr was applied, even if still no complete closure was observed.

MTT proliferation assays

MTT assays results are shown in Table 2. Nickel (10^{-5} M) led to an increase in cell proliferation of $\approx 82\%$ compared to the control after 48 h of incubation. In addition Cr (10^{-6} M) caused a decrease of $\approx 11\%$ compared to the control, at the same time. The data shown are the mean values of three independent experiments, each performed in triplicate.

Real-time PCR

TLR, MMP, and ICAM-1 expression

Real time-PCR showed a down-regulation of TLR-2, -4, and -9 mRNA chromium-induced (Figure 4a) among viable, activated cells. A different trend was observed if Cr persisted in the medium during the healing process. In fact, after the initial down-regulation, the persistence of Cr led to increases in TLR(s) mRNA over time (Figure 4b). TLR-7 mRNA, instead, was up-regulated at the beginning of the healing process, with significant differences if Cr persisted. These values returned to near or just below basal levels at the end of the incubation period (Figures 5a and 5b).

Matrix metalloproteinases were modulated during wound closure. Cr pre-treatment moderately increased

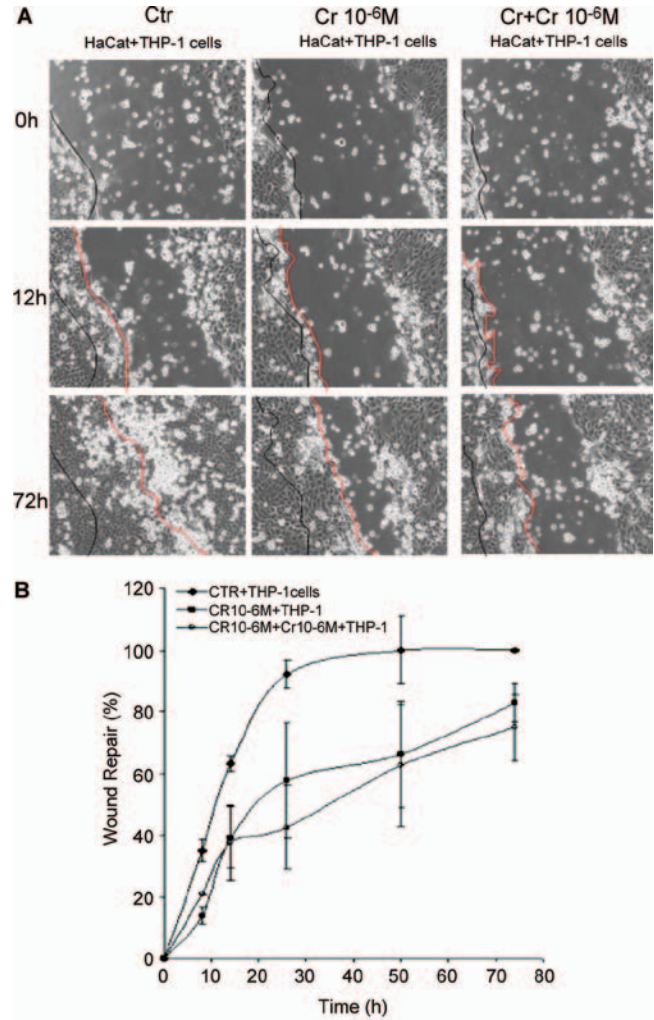


Figure 3. Effects of Cr and Cr + Cr (10^{-6} M) on scratch wound healing of HaCat cell confluent monolayer in the presence of THP-1 cell (1:3 monocytes:keratinocytes). (a) Phase-contrast time-lapse of HaCat cells treated with Cr, then scratched before applying THP-1 cells. Figures were taken at 0, 12, and 72 h after scratching. Monocytes, visible as bright cells in suspension, were mostly recruited to scratch borders and accelerated wound closure in a controlled acceleration of the healing process. The presence of THP-1 cells helped to close the wound where Cr was applied, even if still incomplete closure was noted. (b) Wound repair (%) of metal-treated cultures (with respect to control). Data represent the mean ($\pm$ SD) of three independent experiments. All groups were significantly different from control ($p < 0.05$).

Table 2. MTT proliferation assay.

	OD at 48 h	% of proliferation vs control cells
Negative control cells ^a	1.364	100%
Positive control ^b	2.041	149%
Nickel (105 M)	2.496	182%
Chromium (106 M)	1.222	89%

OD, Optical Density reading at a wavelength of 570/655 nm.

^aCells maintained in serum-free medium and ^b those maintained in medium with 10% FCS.

MMP-2 over time, while this effect was much more evident for MMP-13 ($p < 0.05$) (Figure 6a). Keeping Cr in the medium, MMP-2 and MMP-13 up-regulation was soon observed (4 h), while declining below basal level

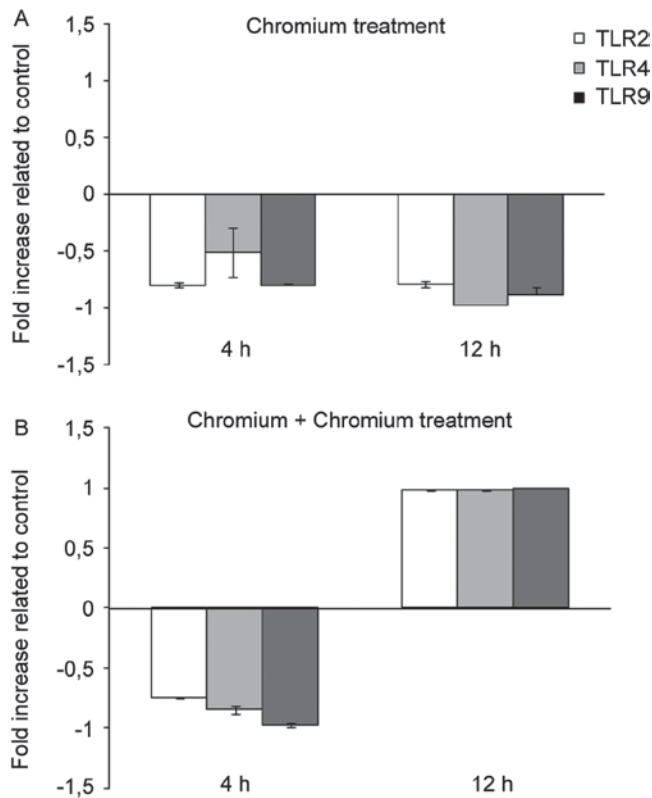


Figure 4. Effects of Cr and Cr + Cr (10^{-6} M) on TLR-2, -4, and -9 expressions from HaCat cells after multiple wounding, at 4 and 12 h. (a) Real time-PCR showing Cr-induced down-regulation of TLR-2, -4, and -9 mRNA. (b) After the initial down-regulation, TLR(s) mRNA levels increased over time if Cr persisted in the medium during the healing process.

on long time (Figure 6b). Pre-treatment with Cr induced up-regulation of ICAM-1 mRNA, but the effects were less evident if the metal persisted in the medium (Figure 7).

Discussion

The detection of the sensitizing potential of chemicals is of great importance to the industry. Allergic sensitization by small molecular weight xenobiotics results in great morbidity. Low molecular allergens include metals such as chromium or nickel. The nature of individual susceptibility to allergic dermatitis might be due to differences in the regulation of the immune system (tolerance vs sensitization) (Curtis et al., 2007). The induction of ACD depends on the production by epidermal cells, within minutes or hours following hapten application, of a rather specific pattern of cytokines resulting in a primary cutaneous immune response (Saint Mezar et al., 2004; Pépin et al., 2007). As a result of the advances in the mechanistic understanding of ACD, *in vitro* assays are growing in importance, as preliminary or alternative systems to expensive, time-consuming, and ethically complex animal studies. In order to elucidate some of the mechanisms of metal-induced wound impairing, we used an *in vitro* model of wound repair with HaCat cells that mimic many properties of normal epidermal keratinocytes (Ermolli et al., 2001; Curtis et al., 2007).

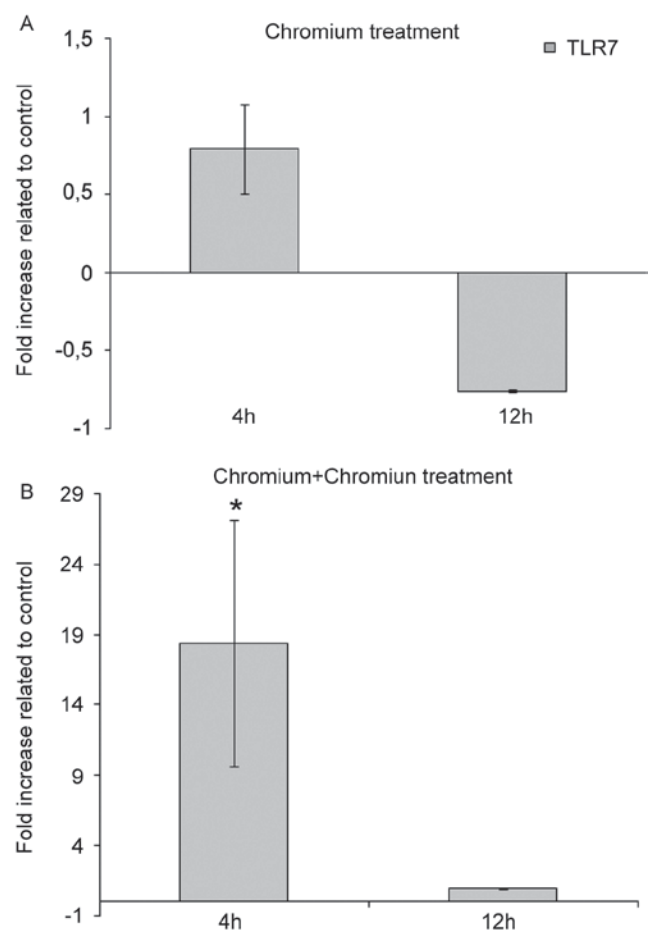


Figure 5. Effects of Cr and Cr + Cr (10^{-6} M) on TLR7 expression from HaCat cells after multiple wounding, at 4 and 12 h. (a and b) Real time-PCR showing up-regulation of TLR-7 mRNA levels at an early portion (i.e., 4 h) of the healing process; there were significant differences if Cr persisted. Values returned to at or below basal levels by the end of the incubation time (i.e., 12 h). Asterisks indicate significant deviations from control (* $p < 0.05$).

Keratinocytes take an active part in the immune response, inflammatory process, and wound healing (Wiegand et al., 2009a). As commonly used in cell-based *in vitro* assays, the chemical test was delivered at the maximum sub/slight cytotoxic concentrations in order to obtain the equivalent of a danger signal to initiate an immune response (Cammarota et al., 2006; Pépin et al., 2007). A time-lapse workstation maintained cells under optimal culture conditions while allowing monitoring and image recording of the scratch closure, providing a level of sensitivity and objectivity that conventional, discontinuous sample observations could not match (Meijering et al., 2008). Control cells recovered the integrity of the monolayer within 72 h, while Cr pre-treatment interrupted the wound closure. On the contrary, nickel accelerated the process. These differences may be explained by the data reporting that nickel ions induce an up-regulation of growth factor receptors expression by keratinocytes and enhanced proliferation of keratinocytes in culture (Ermolli et al., 2001; Thierse et al., 2005; Cammarota et al., 2006; Curtis et al., 2007). Persistence of

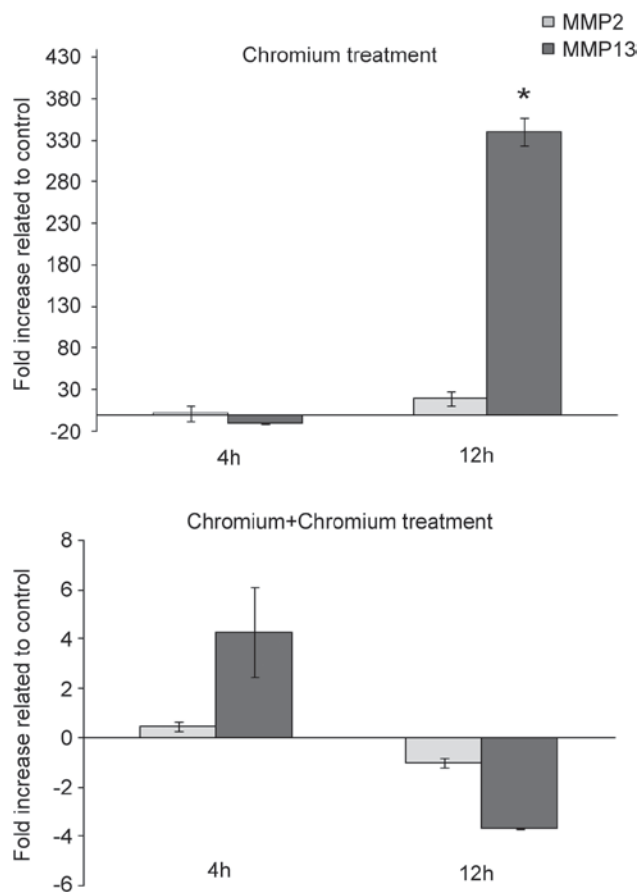


Figure 6. Effects of Cr and Cr + Cr (10^{-6} M) on MMP-2 and MMP-13 expression from HaCat cells after multiple wounding, at 4 and 12 h. (a) Real-time PCR showing that Cr pre-treatment moderately increased MMP-2 mRNA levels over time; this effect was more evident for MMP-13 mRNA. (b) Keeping Cr in the medium, MMP-2 and MMP-13 mRNA up-regulation was soon observed (at 4 h), declining thereafter (by 12 h) to below basal levels. Asterisks indicate significant deviations from control (* $p < 0.05$).

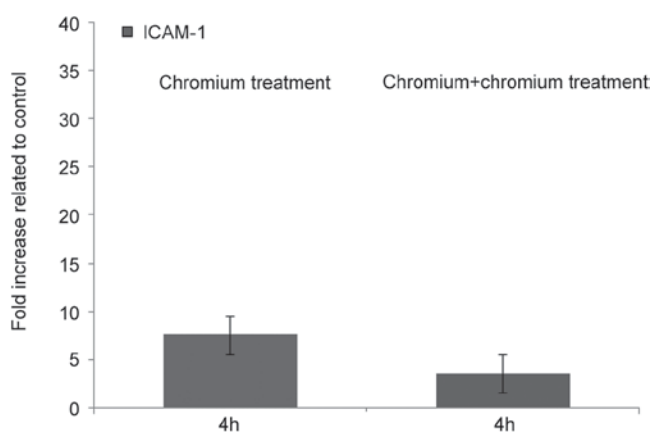


Figure 7. Effects of Cr and Cr + Cr (10^{-6} M) on ICAM-1 expression from HaCat cells after multiple wounding, at 4 h. Real-time PCR showing that Cr pre-treatment induced up-regulation of ICAM-1 mRNA levels. Effects were less evident if Cr persisted in the medium.

Cr during the healing process only slightly worsened the scenario. This phenomenon could be related to findings *in vivo* where it is a peculiarity of chromate contact allergy

that the dermatitis may then become indolent irrespective of whether contact is avoided (Curtis et al., 2007).

HaCat cells provide both the microenvironment, which is relevant for chemical-induced sensitization and the necessary signals for the innate immune system (Bonifas et al., 2010). The involvement of TLRs in metal-induced ACD has never been completely clarified. The expression of TLR-2, -4, and -9 mRNAs was down-regulated when Cr was removed before wounding. Takagi et al. (2007) also demonstrated that mRNA levels of TLR-4 and TLR-9 were decreased after titanium particle stimulation *in vitro*, suggesting that the rapidly responsive but potent TLR system is tightly controlled via some negative feedbacks so as to limit excessive harmful by-stander cell/tissue injury (Khabar, 2007; Wiegand et al., 2009a). In addition, TLR-2, -4, and -9 transcript reduction could provide evidence that abolishing innate immune responses at early time (i.e., 4 h) may be a crucial step for the development of a lesion. Twelve hours after wounding, if Cr persisted, we observed a slight up-regulation of TLR-2, -4, and -9, an initial (immune) response most likely due to an accumulation of endogenous ligands and self-molecules (Min-Fu, 2006). An additional source of TLR-activating molecules could be chemically-modified nucleic acids, for example, formed as a result of metal incorporation in cell nuclei.

Multiple pathways may break tolerance through the activation of nucleic acid-sensing TLR-7 and TLR-9 (Deane and Bolland, 2006). TLR-7 is predominantly located within the endosomal compartments to sense pathogen-derived RNA and DNA motifs generated intracellularly, and self RNA (Köllisch et al., 2005; Smiths et al., 2008). TLR-7 activation culminates in the induction of pro-inflammatory mediators and in the up-regulation of co-stimulatory and adhesion molecules and enhanced migration to boost the immune system (Smiths et al., 2008). TLR-7 mRNA was only slightly increased by Cr pre-treatment in the short-term. Keeping Cr in the culture medium, we observed enhanced TLR-7 mRNA expression, with levels returning close to basal values after 12 h of exposure. The genotoxicity of Cr is characterized by damage to DNA bases and the sugar-phosphate backbone, through direct Cr-DNA interactions or indirect (i.e., via reactive oxygen species-induced) mechanisms (O'Brien et al., 2003). As such, chromatin complexes released from damaged cells contain nucleic acid that may engage TLR-3 and/or TLR-7. The extent of DNA damage induced by Cr can result in a transient cell cycle checkpoint arrest, terminal cell cycle blockade, or apoptosis (Cammarota et al., 2006). As well as induction of innate and cellular immune response, TLR-7 signaling may play a role in eliminating dysplastic or neoplastic epithelial cells triggering apoptosis (Meyer et al., 2003).

In vivo, Cr may affect T_H1 and T_H2 responses in a manner that leads to a deviation of the host immune system towards a T_H1 type. Given that TLR-4, TLR-7, and TLR-9 have been seen as important driving forces of TLR-mediated T_H1 responses, one may argue that the effects of Cr on these types of responses may arise, in part, via TLR-7 activation

(Netea et al., 2005). TLR modulation in the experimental model here was further conditioned by the up-regulation of ICAM-1 mRNA. ICAM-1 is present on the surface of a variety of cell types (including keratinocytes) and is involved in cell-cell and cell-matrix adhesive interactions (Huang et al., 2004). ICAM-1 up-regulation could be related to a number of inflammatory mediators/[soluble] products (including IL1 β and TNF α) that are released as a result of TLR activation. ICAM-1 plays a pivotal role in monocytes adhesion to epithelial cells, facilitating migration through modulation of MMPs (Huang et al., 2004).

Re-epithelialization is related to a correct distribution of MMPs within a wound's micro-environment. In exudates of non-healing wounds, much higher concentrations of MMP have been found than in normal wounds, along with a significantly lower amount of their specific inhibitors (i.e., TIMPs) (Wiegand et al., 2009b). Heat shock proteins and extracellular matrix components, such as fibronectin and hyaluronan released during tissue injury, have been reported to activate the immune system through TLR(s) (Rahman et al., 2009). HaCat cells treated with Cr exhibited a slight increase of MMP-2 mRNA but a dramatic increase of MMP-13 mRNA, especially over time. MMP-2 and MMP-13 are interstitial collagenases, cleaving fibrillar collagen in skin homeostasis. Previous reports have described the contribution of MMP-13 to wound repair in several tissues, acting on keratinocyte migration, angiogenesis, and contraction during wound healing (López-Rivera et al., 2005; Hattori et al., 2009). MMP-13 expression is strongly up-regulated shortly after injury and declines when re-epithelialization is completed; as such, our observations here may be an early signal of revitalization by keratinocytes.

Overall, epithelial cells of the skin, which are under constant attack from external agents, have regulatory mechanisms to controlling inflammation when the epithelial barrier is intact. On the contrary, they need to up-regulate the protective immune response when the epithelial barrier is breached and activate immune-competent cells (Miller et al., 2005). Keeping Cr in the culture medium increased the levels of MMP-2 and MMP-13 transcripts; in contrast, these were drastically down-regulated with longer (i.e., 12 h) incubations. These results may be related to the loss of proliferation and migration ability of keratinocytes.

Skin wound healing is mediated by inflammatory cell infiltration that is highly regulated by various adhesion molecules such as ICAM-1 (CD54) (Tomita et al., 2009). Mice lacking ICAM-1 delayed skin wound healing that was associated with decreased infiltration of neutrophils and macrophages (Yukami et al., 2007). Chromium pre-treatment may induce ICAM-1 mRNA up-regulation of the inflammatory stage of the healing process. The persistence of the chemical insult, however, exerted less effect. In agreement with Pritchard et al. (2000), Cr(VI) induced increases in adhesive properties of the endothelium, thereby impacting on the role of these cells in the initiation/progression of tissue injury through an increased recruitment of pro-inflammatory leukocytes.

The use of an *in vitro* single cell culture system provided defined cultured conditions, avoiding the complex homeostatic mechanisms that occur *in vivo*, and allowed us the direct measurements of TLR involvement in chromium-related toxicity and wound impairing. Furthermore, to reproduce the complex biology of the immune response *in vivo*, besides non-infectious danger signaling, co-culture of keratinocytes with monocytes (THP-1) have been developed. The THP-1 monocytic cell line showed as a response to sensitizers as dendritic cells and primary cultures (Schreiner et al., 2007). Time-lapse monitoring of the wound closure showed that THP-1 cells moved on the borders of the lesion probably due to high concentration of IL-1 β and ICAM-1 released by damaged cells. In addition, the presence of monocytes seems to exert beneficial effects in terms of rapidity of restoring the integrity of the monolayer. Moreover, it could be hypothesized that THP-1 cells physically contribute to fill the gap in control cell cultures and partially in the treated cell systems.

Conclusions

The approach used in the studies reported here might be useful for helping to understand various normal wound-healing events as well as those occurring under pathological conditions. The evolving understanding of the pathophysiology of contact dermatitis will eventually allow for novel and more specific pharmacologic alternatives to be derived for treatment of the disease. Moreover, the experimental model employed here could potentially be used for the screening of novel molecules of therapeutic/preventive interest for the treatment of skin lesions that arise as a result of allergic contact dermatitis.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

- Bianchi, M. E. 2007. DAMPs, PAMPs, alarmins: All we need to know about danger. *J. Leukocyte Biol.* 81:1–5.
- Bonifas, J., Hennen, J., Dierolf, D., Guerreiro, N., and Weltzien, H. U. 2010. Evaluation of cytochrome P450-1 (CYP1) and N-acetyltransferase 1 (NAT1) activities in HaCat cells: Implications for the development of *in vitro* techniques for predictive testing of contact sensitizers. *Toxicology* 24:973–980.

- Cammarota, M., Lamberti, M., Masella, L., Galletti, P., De Rosa, M., Sannolo, N., and Giuliano, M. 2006. Matrix metalloproteinases and their inhibitors as biomarkers for metal toxicity in vitro. *Toxicol. In Vitro* 20:1125–1132.
- Curtis, A., Morton, J., Balafa, C., MacNeil, S., Gawkrödger, D. J., Warren, N. D., and Evans, G. S. 2007. The effects of nickel and chromium on human keratinocytes: Differences in viability, cell associated metal and IL-1 α release. *Toxicol. In Vitro* 21:809–819.
- Deane, J. A., and Bolland, S. 2006. Nucleic acid-sensing TLRs as modifiers of autoimmunity. *J. Immunol.* 177:6573–6578.
- Ermolli, M., Menne, C., Pozzi, G., Serra, M. A., and Clerici, L. A. 2001. Nickel, cobalt and chromium-induced cytotoxicity and intracellular accumulation in human HaCat cells. *Toxicology* 159:23–31.
- Forte, G., Petrucci, F., and Bocca, B. 2008. Metal allergens and growing significance: Epidemiology, immunotoxicology, strategies for testing and prevention. *Inflam. Allergy Drug Targets* 7:145–162.
- Fotos, J. S., Patel, V. P., Karin, N. J., Temburni, M. K., Koh, J. T., and Galileo, D. S. 2006. Automated time-lapse microscopy and high-resolution tracking of cell migration. *Cytotechnology* 51:7–19.
- Gaspari, A. A. 2005. Multiple pathways driving IgE production and chronic dermatitis in mice: A model for atopic dermatitis? *J. Invest. Dermatol.* 124:119–124.
- Gill, S. E., and Parks, W. C. 2008. Metalloproteinases and their inhibitors: Regulators of wound healing. *Int. J. Biochem. Cell. Biol.* 40:1334–1347.
- Hartenstein, B., Thilo Dittich, B., Stickens, D., Stickens, D., Heyer, B., Vu, T. H., Teurich, S., Schorpp-Kistner, M., Werb, Z., and Angel, P. 2006. Epidermal development and wound healing in matrix metalloproteinase-13-deficient mice. *J. Invest. Dermatol.* 126:486–496.
- Hattori, N., Mochizuki, S., Kishi, K., Nakajima, T., Takaishi, H., D'Armiento, J., and Okada, Y. 2009. MMP-13 plays a role in keratinocyte migration, angiogenesis, and contraction in mouse skin wound healing. *Am. J. Pathol.* 175:533–546.
- Huang, W. C., Chan, S. T., and Yang, T. L. 2004. Inhibition of ICAM-1 gene expression, monocyte adhesion, and cancer cell invasion by targeting IKK complex: Molecular and functional study of novel α -methylene- γ -butyrolactone derivatives. *Carcinogenesis* 25:1925–1934.
- Hussein, M. R., Abdel-Magid, W. M., Saleh, R., and Nada, E. 2009. Phenotypical characteristics of the immune cells in allergic contact dermatitis, atopic dermatitis, and pityriasis rosea. *Pathol. Oncol. Res.* 15:73–79.
- Khabar, K. S. 2007. Rapid transit in the immune cells: The role of mRNA turnover regulation. *J. Leukocyte Biol.* 81:1335–1344.
- Köllisch, G., Kalali, B. N., Voelcker, V., Wallich, R., Behrendt, H., Ring, J., Bauer, S., Jakob, T., Mempel, M., and Ollert, M. 2005. Various members of the Toll-like receptor family contribute to the innate immune response of human epidermal keratinocytes. *Immunology* 114:531–541.
- Lan, R., Geng, H., Hwang, Y., Mishra, P., Skloss, W. L., Sprague, E. A., Saikumar, P., and Venkatachalam, M. 2010. A novel wounding device suitable for quantitative biochemical analysis of wound healing and regeneration of cultured epithelium. *Wound Repair Regen.* 18:159–167.
- López-Rivera, E., Lizarbe, T. R., and Martínez-Moreno, M. 2005. Matrix metalloproteinase-13 mediates nitric oxide activation of endothelial cell migration. *Proc. Natl. Acad. Sci. USA* 102:3685–3690.
- Meijering, E., Smal, I., Dzyubachyk, O., and Olivo-Marine, J. C. 2008. Time-lapse imaging. In: *Microscope Image Processing* (Wu, Q., Merchant, F.A., and Castleman, K. R., Eds.). London: Elsevier Academic Press, pp. 401–440.
- Meyer, T., Nindi, I., Schmook, T., Ulrich, C., Sterry, W., and Stockfleth, E. 2003. Induction of apoptosis by Toll-like receptor-7 agonist in tissue cultures. *Br. J. Dermatol.* 149:9–13.
- Miller, L. S., and Modlin, R. L. 2007. Toll-like receptors in the skin. *Semin. Immunopathol.* 29:15–26.
- Miller, L. S., Sørensen, O. E., Liu, P. T., Jalian, H. R., Eshtiahpour, D., Behmanesh, B. E., Chung, W., Starner, T. D., Kim, J., Sieling, P. A., Ganz, T., and Modlin, R. L. 2005. TGF α regulates TLR expression and function on epidermal keratinocytes. *J. Immunol.* 174:6137–6143.
- Min-Fu, T. 2006. Toll-like receptors, inflammation and cancer. *Semin. Cancer Biol.* 16:32–37.
- Netea, M. G., van der Meer, J. W., Suttmüller, R. P., Adema, G. J., and Kullberg, B. J. 2005. From the T_H1/T_H2 paradigm towards a toll-like receptor/T-helper bias. *Antimicrob. Agents Chemother.* 49:3991–3996.
- O'Brien, T. J., Ceryak, S., and Patierno, S. 2003. Complexities of chromium carcinogenesis: Role of cellular response, repair and recovery mechanisms. *Mutat. Res.* 533:3–36.
- Pépin, E., Goutet, M., and Ban, M. 2007. Murine bone marrow-derived dendritic cells as a potential in vitro model for predictive identification of chemical sensitizers. *Toxicol. Lett.* 175:89–101.
- Perfetto, B., Lamberti, M., Giuliano, M. T., Canozo, N., Cammarota, M., and Baroni, A. 2007. Analysis of the signal transduction pathway of nickel-induced matrix metalloproteinase-2 expression in the human keratinocytes in vitro: Preliminary findings. *J. Cutan. Pathol.* 34:441–447.
- Pritchard, K. A., Ackerman, A., and Kalyanaraman, B. 2000. Chromium (VI) increases endothelial cell expression of ICAM-1 and decreases nitric oxide activity. *J. Environ. Pathol. Toxicol. Oncol.* 19:251–260.
- Rahman, A. H., Taylor, D. K., and Turka, L. A. 2009. The contribution of direct TLR signaling to T-cell responses. *Immunol. Res.* 45:25–36.
- Saint Mezard, P., Rosieres, A., Krasteva, M., Berard, F., Dubois, B., Kaiserlian, D., and Nicolas, J. F. 2004. Allergic contact dermatitis. *Eur. J. Dermatol.* 14:284–295.
- Schmittgen, T. D., Zakrajsek, B. A., Mills, A. G., Gorn, V., Singer, M. J., and Reed, M. W. 2000. Quantitative reverse transcription-polymerase chain reaction to study mRNA decay: Comparison of endpoint and real-time methods. *Anal. Biochem.* 285:194–204.
- Schreiner, M., Peiser, M., Briechele, D., Stahlmann, R., Zuberbier, T., and Wanner, R. 2007. A loose-fit co-culture of activated keratinocytes and dendritic-cell related cells for prediction of sensitizing potential. *Allergy* 62:1419–1428.
- Smits, E. L., Ponsaerts, P., Berneman, Z. N., and van Tendeloo, V. G. 2008. The use of TLR7 and TLR8 ligands for the enhancement of cancer immunotherapy. *The Oncologist* 13:859–875.
- Sugita, K., Kabashima, K., Atarashi, K., Shimauchi, T., Kobayashi, M., and Tokura, Y. 2006. Innate immunity mediated by epidermal keratinocytes promotes acquired immunity involving Langerhans cells and T-cells in the skin. *Clin. Exp. Immunol.* 147:176–183.
- Takagi, M., Tamaki, Y., Hasegawa, H., Takakubo, Y., Kontinen, L., Tiainen, V. M., Lappalainen, R., Kontinen, Y. T., and Salo, J. 2007. Toll-like receptors in the interface membrane around loosening total hip replacement implants. *J. Biomed. Mat. Res.* 81:1017–1026.
- Thierse, H. J., Gämderinger, K., Junkes, C., Guerreiro, N., and Weltzien, H. U. 2005. T-Cell receptor (TCR) interaction with haptens: Metal ions as non-classical haptens. *Toxicology* 209:101–107.
- Tomita, H., Iwata, Y., Ogawa, F., Komura, K., Shimizu, K., Yoshizaki, A., Hara, T., Muroi, E., Yanaba, K., Bae, S., Takenaka, M., Hasegawa, M., Fujimoto, M., and Sato, S. 2009. P-Selectin glycoprotein ligand-1 contributes to wound healing predominantly as a P-selectin ligand and partly as an E-selectin ligand PSGL-1 regulates cutaneous wound healing. *J. Invest. Dermatol.* 129:2059–2067.
- Wiegand, C., Abel, M., Ruth, P., and Hippler, U. C. 2009a. HaCat keratinocytes in co-culture with *Staphylococcus aureus* can be protected from bacterial damage by polihexanide. *Wound Repair Regen.* 17:730–738.
- Wiegand, C., Heinze, T., and Hippler, U. C. 2009b. Comparative in vitro study on cytotoxicity, anti-microbial activity, and binding capacity for pathophysiological factors in chronic wounds of alginate and silver-containing alginate. *Wound Repair Regen.* 17:511–521.
- Yukami, T., Hasegawa, M., Matsushita, Y., Fujita, T., Matsushita, T., Horikawa, M., Komura, K., Yanaba, K., Hamaguchi, Y., Nagaoka, T., Ogawa, F., Fujimoto, M., Steeber, D. A., Tedder, T. F., Takehara, K., and Sato, S. 2007. Endothelial selectins regulate skin wound healing in cooperation with L-selectin and ICAM-1. *J. Leukocyte Biol.* 82:519–531.